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Coronavirus Proteins: Structure and Function of the Oligosaccharides of the Avian Infectious Bronchitis Virus Glycoproteins

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The recent finding that the E1 glycoproteins of murine coronaviruses contain only O-linked oligosaccharides suggested that this unusual modification might be a distinguishing feature of coronaviruses and might play an essential role in the life cycle of this family of viruses. To examine these possibilities, we analyzed the oligosaccharide moieties of the membrane proteins of the avian coronavirus infectious bronchitis virus. In addition, we determined the effect of inhibiting the glycosylation of these proteins on viral maturation and infectivity. Infectious bronchitis virus virions contain nine proteins. Four of these proteins, GP36, GP31, GP28, and P23, are closely related structurally and appear to be homologous to the E1 proteins of murine coronaviruses. We found that the oligosaccharides of GP31 and GP28 could be removed with endoglycosidase H and that neither of these glycoproteins was detectable in tunicamycin-treated cells. These two results indicated that GP31 and GP28 contain N-linked oligosaccharides. Therefore, O-linked oligosaccharides are not a universal feature of the small coronavirus membrane glycoproteins. Tunicamycin inhibited glycosylation of all of the viral glycoproteins but did not inhibit production of virions by infectious bronchitis virus-infected cells. The virions released by these cells contained only the three non-glycosylated viral proteins P51, P23, and P14. These particles were not infectious. Therefore, it appears that glycosylated infectious bronchitis virus polypeptides are not required for particle formation. However, the viral glycoproteins are apparently indispensible for viral infectivity.

Coronaviruses are a recently characterized group of enveloped viruses which contain large single-stranded RNA genomes of messenger polarity (8, 14). We have been studying the replication of the avian coronavirus infectious bronchitis virus (IBV) in tissue culture. In this paper we analyze the glycosylation of viral membrane polypeptides.

Coronavirus membrane proteins are of special interest because the E1 proteins of murine coronaviruses are glycosylated in an unusual way. The majority of the viral glycoproteins characterized to date contain only N-linked oligosaccharides (5). In contrast, the E1 glycoproteins contain O-linked oligosaccharides exclusively (4, 6, 11). Therefore, O-linked oligosaccharides might be a distinguishing characteristic of coronaviruses and play an essential role in coronavirus multiplication. To investigate this possibility, we characterized the oligosaccharide moieties of the IBV glycoproteins.

IBV virions contain nine proteins (2, 15). All but three of these proteins (P51, P23, and P14) are glycosylated. Two glycoproteins, GP90 and GP84, probably form the viral peplomers (2), the rounded surface projections for which the coronaviruses are named. The other three major glycoproteins belong to a family of very closely related proteins, which we have designated the P23 family. This family consists of the most abundant virion protein (GP31), two less abundant glycoproteins (GP36 and GP28), and the non-glycosylated protein P23. We have shown that the differences in the molecular weights of these proteins are caused by structural differences in their amino-terminal domains (15). Since these domains contain oligosaccharides, we have suggested that GP36, GP31, and GP28 might simply be glycosylated forms of P23 (15). Thus, IBV virions appear to contain three different glycosylated polypeptides, GP90, GP84, and the glycoproteins of the P23 family.

The glycoproteins of the murine coronaviruses mouse hepatitis virus A59 and JHM are similar to those of IBV in several respects. Like IBV, murine coronaviruses contain a heteroge-

neous family of small membrane proteins, which are collectively designated the E1 proteins (3, 11, 13, 19). All but the smallest of the E1 polypeptides are glycosylated; it is these proteins which contain only O-linked oligosaccharides. The murine coronaviruses also contain high-molecular-weight glycoproteins designated E2 proteins, which appear to comprise the viral surface projections (20). Unlike E1 proteins, the E2 proteins contain N-linked oligosaccharides (6). The E2 proteins can be isolated in two forms, which have molecular weights of 90,000 and 180,000. The similarity of the peptide maps of these two proteins (20) suggests that they are monomeric and dimeric forms of a single polypeptide. Thus, in contrast to IBV, only a single large polypeptide has been identified in murine coronavirus particles.

Tunicamycin, which inhibits the glycosylation of polypeptides on asparagine residues (7, 18, 21, 23), was found not to inhibit production of murine coronavirus particles. However, the virions released from treated cells were noninfectious and lacked E2 proteins (4, 6, 11). This showed that the E2 proteins are essential for infectivity, but may not be required for production of virions. Since the synthesis of O-linked oligosaccharides is not affected by tunicamycin, these experiments did not address the question of whether glycosylation is necessary for the function of the E1 proteins. Therefore, the importance of glycosylation of the small coronavirus glycoproteins (P23 family or E1 proteins) remained to be tested.

In this work we examined the glycosylation of IBV polypeptides to determine whether the P23 family glycoproteins of IBV contain exclusively O-linked oligosaccharides, whether the P23 family proteins all contain exactly the same core polypeptide, and whether glycosylation of coronavirus proteins is necessary for virion formation and for the infectivity of viral particles.

MATERIALS AND METHODS

Virus and cells. The Beaudette strain (strain 42) of IBV was propagated in primary chicken embryo kidney (CEK) cells as described previously (15, 16). Cells were incubated at 37 or 38.5°C.

Labeling and purification of virion proteins. Virions were labeled biosynthetically with [^{35}S]methionine and purified as described previously (15). Portions of the gel-purified protein samples prepared for peptide mapping as described in the accompanying paper (17) were precipitated with trichloroacetic acid and digested with endoglycosidase H.

Labeling and purification of virions from tunicamycin-treated cells. CEK cell cultures in 60-mm dishes were infected as described in the accompanying paper (17). At the end of the adsorption period, the inoculum was replaced with Dulbecco-Vogt modified Eagle medium containing one-tenth the normal amount of methionine and 2% calf serum dialyzed against saline. After 4 h the medium was replaced with 2 ml of methionine-free Dulbecco-Vogt modified Eagle medium containing 2% dialyzed calf serum and 50 μCi of [^{35}S]methionine (1,200 Ci/mmol; Amersham Corp.). Tunicamycin (Calbiochem-Behring Corp.) dissolved in dimethyl sulfoxide at a concentration of 100 μg/ml was added to treated cultures to yield a final concentration of 1 μg/ml. The drug was added at the end of the adsorption period.

Virions were harvested 8.5 h postadsorption. The harvested medium was centrifuged for 2 min in a Brinkmann microcentrifuge, and 0.6-ml portions of the supernatant were applied to 11.0-ml 20% (wt/vol) to 55% (wt/wt) sucrose gradients prepared in 50 mM Tris (pH 7.4)–100 mM sodium chloride–1 mM EDTA (disodium salt). The gradients were centrifuged in an SW41 rotor at 150,000 × *g* for 17 h at 4°C and fractionated, and the virus-containing peaks were located and quantified by scintillation spectrometry. Virions were concentrated from peak fractions by sedimentation at 85,800 × *g* for 3 h at 4°C in an SW50.1 rotor. The pelleted virions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Analysis of intracellular proteins. CEK cell cultures in 60-mm dishes were infected with IBV and labeled with [^{35}S]methionine as described in the accompanying paper (17). Cells were lysed in RIPA buffer, and immunoprecipitations were performed as described previously (12) by using the rabbit anti-IBV serum described in the accompanying paper (17). This serum recognizes GP155, GP84, P51, and the P23 family proteins but does not react well with GP90. To examine synthesis of intracellular proteins in the presence of tunicamycin, the drug was added at the end of the adsorption period to a final concentration of 1 μg/ml. Cells were labeled with [^{35}S]methionine (125 μCi per 60-mm culture dish) at 6 h postadsorption and lysed for immunoprecipitation at 7.7 h postadsorption.

Endoglycosidase H digestion. Endoglycosidase H (34.5 IU/mg) was obtained from Health Research Inc. The procedures for digestion of immunoprecipitated proteins, digestion of total virion proteins, and digestion of gel-purified virion proteins differed slightly. Immunoprecipitates were prepared from RIPA lysates of infected cells but were not suspended in electrophoresis sample buffer. The pellets were washed once in 10 mM Tris (pH 6.8)–0.1% Nonidet P-40, suspended in 50 mM Tris (pH 6.8), containing 1% SDS, and incubated at 100°C for 30 s, and the bacteria were pelleted. Portions (9.5 μl) were transferred to tubes which contained 10 μl of 150 mM sodium citrate (pH 5.5) and the appropriate amount of endoglycosidase H, and the preparations were incubated for 17 h at 37°C. Then 4 μl of 10% SDS was added, and the samples were incubated at 100°C for 30 s. The samples were precipated with acetone and suspended in electrophoresis sample buffer. For digestion of unfractionated virion proteins, virus-containing pellets were suspended in 50 mM Tris-hydrochloride (pH 6.8)–1% SDS and treated as just described. Trichloroacetic acid precipitates of gel-purified proteins were treated in the same way and incubated for 26 h. However, these reactions were terminated by adding twofold-concentrated electrophoresis sample buffer; the acetone precipitation step was omitted.

SDS-polyacrylamide gel electrophoresis. Samples

were suspended in electrophoresis sample buffer (5 mM sodium phosphate, pH 7.0, 2% SDS, 0.1 M dithiothreitol, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue), boiled for 30 s, and analyzed in discontinuous 15% acrylamide–0.09% bisacrylamide gels as described previously (12). Analytical gels were 14 cm long by 1 mm thick. Gels were prepared for fluorography (1) and exposed to Kodak X-Omat R film at −70°C.

RESULTS

Analysis of the oligosaccharides of the P23 family glycoproteins. Since the P23 family glycoproteins can be labeled with mannose (15), we suspected that they bore N-linked oligosaccharides and therefore differed from the murine coronavirus E1 proteins. We used two ap-

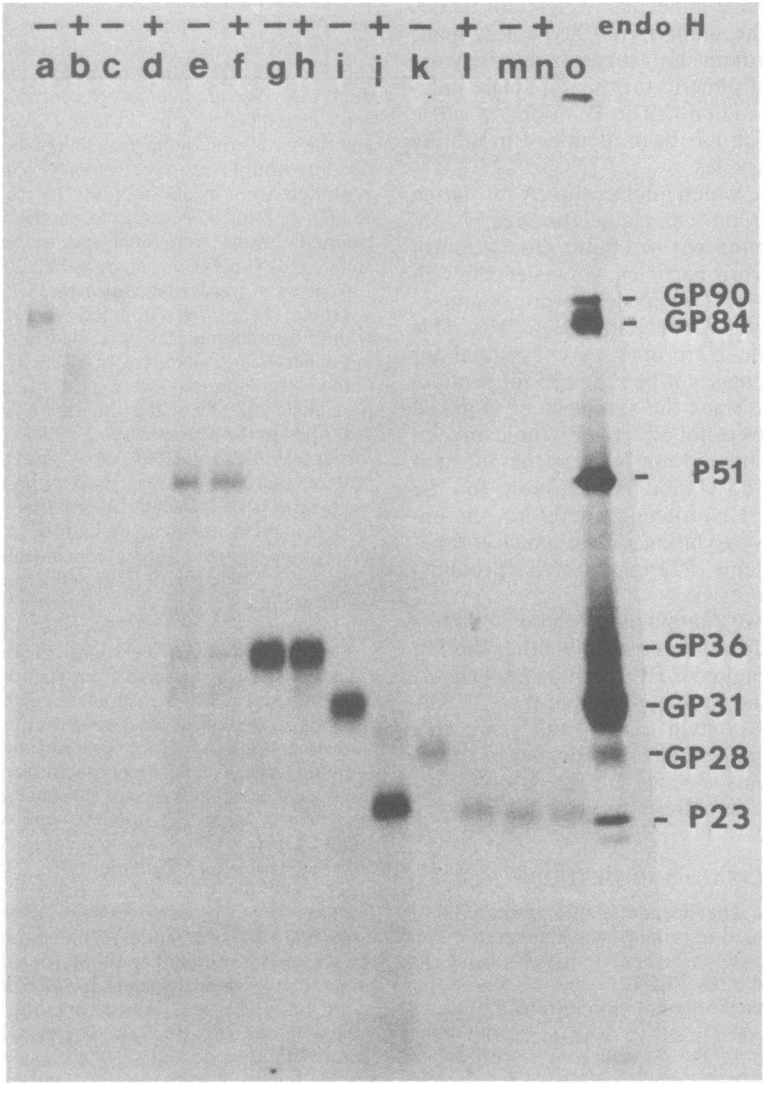

FIG. 1. Endoglycosidase H digestion of gel-purified virion proteins. Virion proteins were fractionated on an SDS-polyacrylamide gel, eluted, and digested with endoglycosidase H (endo H) at a concentration of 1 μg/ml for 26 h at 37°C. The products were analyzed by SDS-polyacrylamide gel electrophoresis. The fluorograph was exposed for 35 days. +, Digested proteins; −, mock-digested proteins. Lane a, GP84, mock digested; lane b, GP84, digested; lane c, GP90, mock digested; lane d, GP90, digested; lane e, P51, mock digested; lane f, P51, digested; lane g, GP36, mock digested; lane h, GP36, digested; lane i, GP31, mock digested; lane j, GP31, digested; lane k, GP28, mock digested; lane l, GP28, digested; lane m, P23, mock digested; lane n, P23, digested; lane o, virions.

proaches to examine this possibility more directly. The first was to determine whether the glycoproteins contain any oligosaccharide sensitive to removal by endoglycosidase H, and the second was to investigate the sensitivity of glycosylation of the P23 family proteins to the drug tunicamycin.

Endoglycosidase H cleaves the *N*,*N*'-diacetylchitobiose group of asparagine-linked oligosaccharides, releasing the bulk of the oligosaccharides and leaving a single *N*-acetylglucosamine residue linked to asparagine (22). Endoglycosidase H does not cleave mature complex N-linked oligosaccharides or O-linked oligosaccharides but efficiently cleaves both simple (high-mannose) N-linked oligosaccharides and the precursors to simple and complex N-linked oligosaccharides (9).

Virion proteins were purified by gel electrophoresis and digested with endoglycosidase H (Fig. 1). The non-glycosylated proteins P51 and P23 were not affected by endoglycosidase H digestion (Fig. 1, lanes e, f, m, and n). This demonstrated that the enzyme preparation was not contaminated with proteases. GP31 and GP28 were cleaved (lanes j and l) to yield a protein just larger than P23. This suggested that P23, GP28, and GP31 contain the same core polypeptide. Since the cleavage products of GP31 and GP28 were almost identical in size to P23, it appeared that GP31 and GP28 bear asparagine-linked oligosaccharides exclusively. The small residual difference in mobility between the GP31 and GP28 digestion products and P23 (see also Fig. 2, lanes e and f) might have resulted from the *N*-acetylglucosamine residues which remained with the polypeptide after endoglycosidase H cleavage. Approximately 3% of GP31 (as determined by densitometry of the fluorograph) was cleaved to yield a protein slightly larger than GP28. This minor component must have contained at least one sensitive (high-mannose) oligosaccharide and a resistant modification. GP36 was entirely resistant to endoglycosidase H digestion (Fig. 1, lane h). GP90 and GP84 were both digested with endoglycosidase H, but the cleavage products were heterogeneous (Fig. 1, lanes b and d).

We estimated the minimum number of high-mannose oligosaccharides linked to IBV proteins by enumerating the partial endoglycosidase cleavage products obtained by digestion with limiting amounts of enzyme (10, 24). Virion proteins from IBV labeled biosynthetically with [^{35}S]methionine were digested with different amounts of endoglycosidase H (Fig. 2, lanes b through e). Most of GP31 was digested at the lowest concentration of the enzyme used. An intermediate product (Fig. 2, lane b, arrowhead), which had a slightly lower apparent molecular weight than GP28, was visible. This intermediate was probably derived from GP31 rather than GP36 or GP28 since GP36 is resistant to cleavage and the intermediate was more abundant than GP28 in the starting material. At higher concentrations of endoglycosidase H (Fig. 2, lanes c through e), the partial product was no longer evident. The difference in size between the major GP31 digestion product (Fig. 2, lane e, arrow) and virion P23 (lane f, arrow) was clear. The minor GP31 cleavage product which was observed in Fig. 1 is indicated with an asterisk in Fig. 2, lane b.

GP84 and GP90 are derived from proteolytic cleavage of a 155-kilodalton (kd) intracellular protein (17). To determine whether this precursor protein is glycosylated, we examined its sensitivity to endoglycosidase H. IBV-specific proteins were immunoprecipitated from infected cell lysates with an antiserum raised against virion proteins (17), and the proteins in the immunoprecipitate were digested with increasing amounts of endoglycosidase H (Fig. 2, lanes g through j). The digestion pattern of the intracellular form of GP31 was identical to that of GP31 obtained from virions (Fig. 2, lanes b through d, h, and i). At least two partial digestion products of the 155-kd protein were visible (Fig. 2, lanes h and i, arrows), demonstrating that this protein contains a minimum of three high-mannose oligosaccharide chains. We also found that synthesis of the protein was inhibited by tunicamycin (see below). These two observations indicate that the 155-kd protein is glycosylated, and we designated this protein GP155.

Effect of tunicamycin on viral protein synthesis. Since the products of endoglycosidase H cleavage of GP31 and GP28 were almost identical in size to P23, it seemed probable that the P23 family proteins contained the same core polypeptide. We used the drug tunicamycin to test this hypothesis rigorously (Fig. 3A). In the presence of this drug polypeptides are not glycosylated on asparagine residues (7). The labeling of P51 was unaltered in tunicamycin-treated cultures (Fig. 3A, lane d), demonstrating that the drug did not have an effect upon synthesis of non-glycosylated viral proteins. GP155, GP84, and GP31 were not detectable in tunicamycin-treated cells (lane d). A protein which comigrated with P23 accumulated in the treated cells to approximately the same extent as GP31 did in untreated infected cultures (lanes b and d). In this experiment a novel 110-kd protein accumulated, which was approximately as abundant as GP155 in untreated cultures. However, this protein was not detected in other experiments. A second novel polypeptide (Fig. 3A, asterisk) was evident in precipitates of tunicamycin-treated infected cultures. This protein was also found in

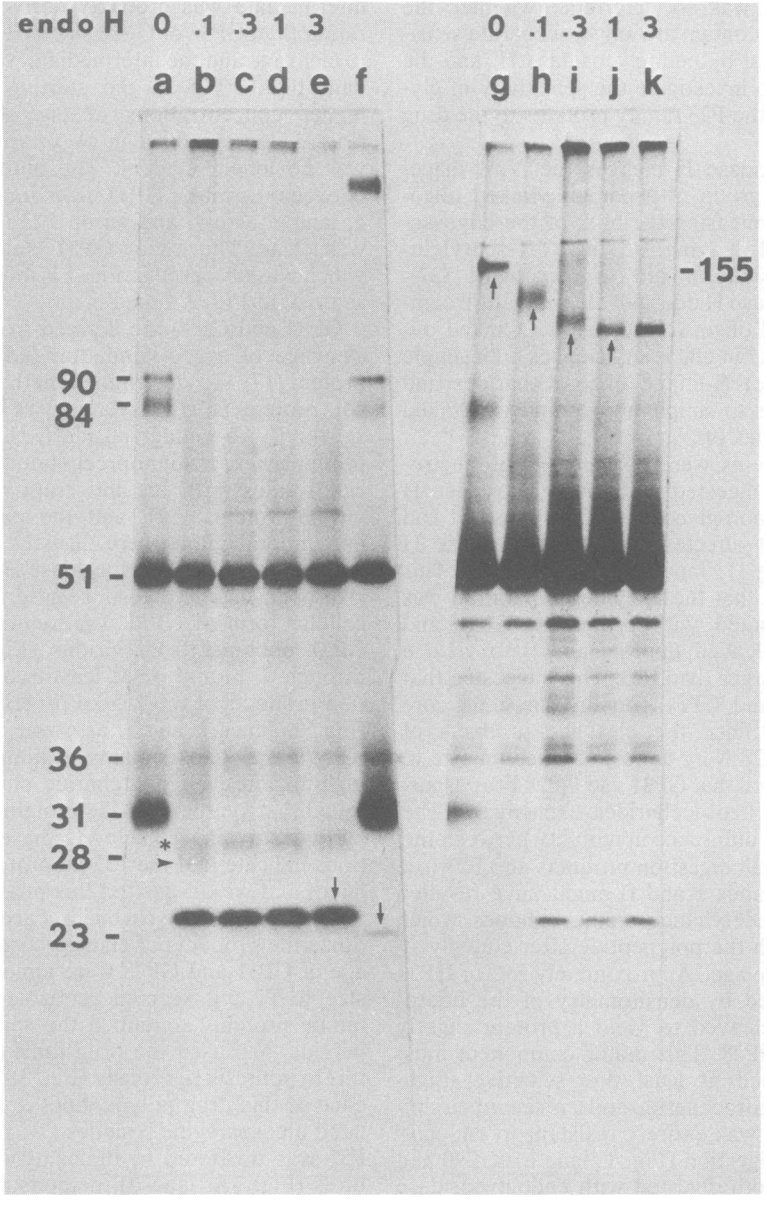

FIG. 2. Partial digestion of virion proteins and intracellular proteins with endoglycosidase H. Virions labeled biosynthetically with [^{35}S]methionine were purified and incubated with endoglycosidase H (endo H) for 17 h at 37°C. [^{35}S]methionine-labeled intracellular proteins were precipitated from RIPA lysates of infected cells by using rabbit anti-IBV serum and incubated with endoglycosidase H under the same conditions. The two sections of this figure are different portions of a single gel. A total of 25,000 cpm was analyzed in each of lanes a through e; these lanes were exposed for 1 day. The remaining portion of the gel was fluorographed for 14 days. The arrows in lanes g through j indicate GP155 and its cleavage products. The other symbols are discussed in the text. Lane a, Virions incubated without endoglycosidase H; lane b, virions incubated with 0.1 μg of endoglycosidase H per ml; lane c, virions incubated with 0.3 μg of endoglycosidase H per ml; lane d, virions incubated with 1.0 μg of endoglycosidase H per ml; lane e, virions incubated with 3.0 μg of endoglycosidase H per ml; lane f, virions, not boiled before electrophoresis; lane g, intracellular proteins incubated without endoglycosidase H; lane h, intracellular proteins incubated with 0.1 μg of endoglycosidase H per ml; lane i, intracellular proteins incubated with 0.3 μg of endoglycosidase H per ml; lane j, intracellular proteins incubated with 1.0 μg of endoglycosidase H per ml; lane k, intracellular proteins incubated with 3.0 μg of endoglycosidase H per ml.

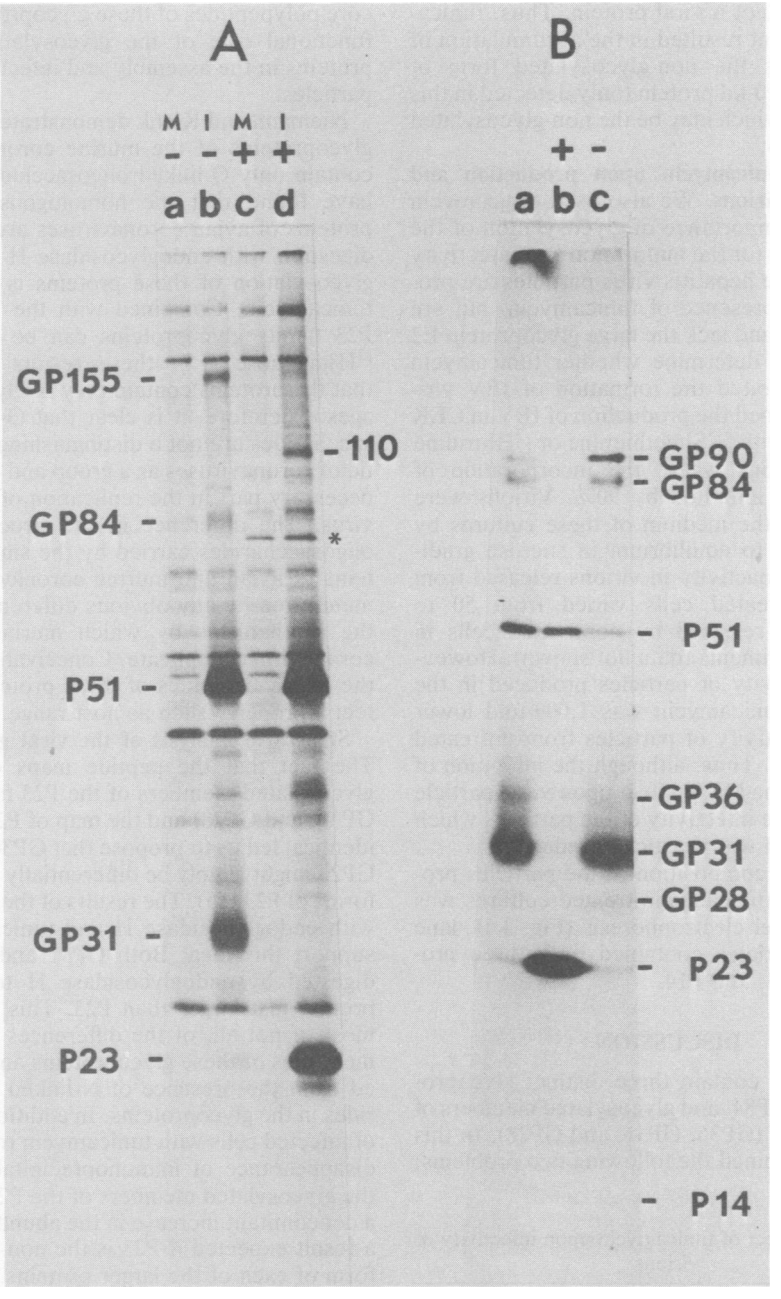

FIG. 3. (A) Effect of tunicamycin on protein synthesis in IBV-infected cells. CEK cells were infected with IBV as described in the text and labeled with [^{35}S]methionine. Tunicamycin was used at a concentration of 1 μg/ml. Immunoprecipitates were prepared from these cultures by using rabbit anti-IBV serum and were analyzed by SDS-polyacrylamide gel electrophoresis. Fluorography was for 7 days. Lane a, Mock infected (M), no tunicamycin; lane b, infected (I), no tunicamycin; lane c, mock infected, with tunicamycin; lane d, infected, with tunicamycin. (B) Protein composition of virions released from tunicamycin-treated cells. CEK cells were infected and labeled with [^{35}S]methionine in the presence or absence of tunicamycin as described in the text. Virions were purified by sedimentation to equilibrium in sucrose gradients, concentrated by pelleting, and analyzed by gel electrophoresis. A total of 1,100 cpm was analyzed in each lane. Fluorography was for 35 days. Lane a, Virions labeled and purified by our standard procedure (15); lane b, virions produced in the presence of tunicamycin; lane c, virions produced in the absence of tunicamycin.

precipitates from uninfected cells (lane c) and therefore was not a viral protein. Thus, tunicamycin treatment resulted in the accumulation of P23, which is the non-glycosylated form of GP31, and a 110-kd protein (only detected in this experiment), which may be the non-glycosylated form of GP155.

Effect of tunicamycin upon production and infectivity of virions. We also used tunicamycin to study the importance of glycosylation of the virion proteins for the maturation and infectivity of IBV. Mouse hepatitis virus particles are produced in the presence of tunicamycin, but are not infectious and lack the large glycoprotein E2 (4, 6, 11). To determine whether tunicamycin treatment inhibited the formation of IBV virions, we examined the production of IBV in CEK cells labeled with [^{35}S]methionine or [^{3}H]uridine under conditions where the incorporation of mannose was inhibited by 90%. Virions were purified from the medium of these cultures by sedimentation to equilibrium in sucrose gradients. The radioactivity in virions released from tunicamycin-treated cells varied from 50 to 200% of that released by nontreated cells in different experiments (data not shown). However, the infectivity of particles produced in the presence of tunicamycin was 1,000-fold lower than the infectivity of particles from untreated cells (Table 1). Thus, although the inhibition of glycosylation had little effect upon virus particle production, the infectivity of the particles which were produced was drastically reduced.

The protein composition of the particles produced by the tunicamycin-treated cultures was analyzed by gel electrophoresis (Fig. 3 B, lane b). These particles contained only three proteins, P51, P23, and P14.

DISCUSSION

IBV virions contain three distinct glycoproteins, GP90, GP84, and glycosylated members of the P23 family (GP36, GP31, and GP28). In this study we examined the following two problems:

TABLE 1. Effect of tunicamycin upon infectivity of virions

Tunicamycin	Total PFU[a]	PFU/cpm[b]
−	$1{,}000 \times 10^4$	570
+	1.4×10^4	0.099

[a] Cells were infected and labeled with [^{35}S]methionine in the presence or absence of tunicamycin as described in the text. Virions were purified by sedimentation to equilibrium in sucrose. The IBV titers in the virus peaks were determined by a plaque assay on CEK cells (21).

[b] Ratio of infectivity to acid-precipitable [^{35}S]methionine activity.

the structures of the oligosaccharides and the core polypeptides of these glycoproteins and the functional role of the glycosylation of these proteins in the assembly and infectivity of virus particles.

Niemann and Klenk demonstrated that the E1 glycoproteins of the murine coronavirus JHM contain only O-linked oligosaccharides (6). We have found that the homologous P23 family proteins of avian coronaviruses are sensitive to digestion with endoglycosidase H and that the glycosylation of these proteins is inhibited by tunicamycin. Combined with the fact that the P23 family glycoproteins can be labeled with [^{3}H]mannose (15), these results demonstrate that the proteins contain only N-glycosidic linkages. Therefore, it is clear that O-linked oligosaccharides are not a distinguishing characteristic of coronaviruses as a group and do not play a necessary part in the replication of this class of virus. The difference in the structures of the oligosaccharides carried by the small glycoproteins of avian and murine coronaviruses is not manifested by any obvious differences between the mechanisms by which murine and avian coronaviruses replicate. Conceivably, however, the oligosaccharides of these proteins could affect a property such as host range.

Structural analysis of the viral glycoproteins. The fact that the peptide maps of the three glycosylated members of the P23 family (GP36, GP31, and GP28) and the map of P23 itself were identical led us to propose that GP36, GP31, and GP28 might simply be differentially glycosylated forms of P23 (15). The results of the experiments with endoglycosidase H and tunicamycin fully support this idea. Both GP31 and GP28 were digested by endoglycosidase H to produce a protein just larger than P23. This showed that most, if not all, of the differences between the mobilities of these glycoproteins and P23 resulted from the presence of N-linked oligosaccharides in the glycoproteins. In addition, treatment of infected cells with tunicamycin resulted in the disappearance of immunoprecipitable forms of the glycosylated members of the P23 family and a concomitant increase in the abundance of P23, a result expected if P23 is the non-glycosylated form of each of the larger proteins.

The sensitivity of mature GP31 and GP28 to endoglycosidase H indicates that these glycoproteins bear simple N-linked oligosaccharides. In contrast, mature GP36 is not affected by the enzyme. Combined with the observation that GP36 can be radiolabeled with mannose and glucosamine (15), this result suggests that GP36 contains complex N-linked oligosaccharides.

Functional analysis of the viral glycoproteins. Murine coronavirus particles are produced efficiently in the presence of tunicamycin (4, 6, 11).

This is not due to an ability to assemble virions in the absence of viral membrane proteins but instead to the fact that the synthesis of the O-linked oligosaccharides which are carried by the E1 glycoproteins is not affected by tunicamycin. However, the virions which are produced are not infectious. The lack of infectivity appears to derive from the absence of E2 proteins, so it appears that E2 proteins are essential for infectivity but dispensable for particle formation. Since a drug that is capable of inhibiting the assembly of O-linked oligosaccharides is not available, the question of whether the glycosylation of the small glycoproteins of the coronaviruses is essential for virion formation was not addressed.

We examined the effect of inhibiting with tunicamycin the addition of N-linked oligosaccharides to the IBV glycoproteins. Noninfectious virions which contained no glycosylated viral proteins were produced at an undiminished rate in IBV-infected cells treated with tunicamycin. The released virions contained only P51, P23, and P14. The absence of GP90 and GP84 in the released noninfectious particles suggests that these compounds are both dispensable for virion production and, by analogy to the murine coronaviruses, essential for viral infectivity. However, non-glycosylated forms of glycoproteins are frequently unusually susceptible to proteolysis (7), and it is conceivable that the large proteins are incorporated into virions and then degraded. The absence of GP36, GP31, and GP28 in these particles demonstrates that glycosylation of P23 is not necessary for particle formation. Whether such glycosylation plays a role in viral infectivity cannot be assessed. The unimpaired incorporation of the P23 family membrane proteins in the form of P23 into particles in the presence of tunicamycin was not unexpected since non-glycosylated P23 is normally incorporated into virions.

The relative abundance of the P23 family proteins in virions correlates with the rates of synthesis and accumulation of these proteins in infected cells. Thus, it does not appear that any particular P23 family protein is preferentially incorporated into virions. The proportion of each protein in virions is a measure of the frequency with which the different forms are generated. The polymorphism of the P23 family proteins is an extreme example of heterogeneity in glycosylation of a single polypeptide. P23 family polypeptides bearing no oligosaccharides (P23), one or two simple N-linked oligosaccharides (GP28), two or three simple oligosaccharides (GP31), simple and complex N-linked oligosaccharides (the minor population of GP31), and apparently only complex oligosaccharides (GP36) are all produced in IBV-infected cells. Thus far it appears that this polymorphism is incidental to the physiology of IBV.

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